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Scientific opinion on Flavouring group evaluation 216 revision 2 (FGE.216Rev2): consideration of the genotoxicity potential of α,β-unsaturated 2-phenyl-2-alkenals from subgroup 3.3 of FGE.19

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Abstract

The EFSA Panel on Food Additives and Flavourings (FAF) was requested to evaluate the genotoxic potential of five flavouring substances from subgroup 3.3 of FGE.19, in the Flavouring Group Evaluation 216 (FGE.216). In FGE.216 and in FGE.216Rev1, the CEF Panel requested additional genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062], the representative for these five substances. New experimental data on [FL-no: 05.062] were provided and are evaluated in the present revision of FGE.216 (FGE.216Rev2). Based on the new data, the Panel concluded that, for all the five substances, the concerns for gene mutations and clastogenicity are ruled out by the negative results observed in an *in vivo* gene mutation assay and in an *in vivo* comet assay, respectively. *In vitro*, [FL-no: 05.062] induced micronuclei through an aneugenic mode of action. The available *in vivo* micronucleus studies were inconclusive and cannot be used to rule out potential aneugenicity of [FL-no: 05.062] *in vivo*. Therefore, the Panel compared the lowest concentration resulting in aneugenicity *in vitro* with the use levels reported for this substance. Based on this comparison, the Panel concluded that the use of the flavouring substance [FL-no: 05.062] at the reported use levels in several food categories would raise a concern for aneugenicity. Based on structural similarity, for the remaining four substances in this FGE [FL-no: 05.099, 05.100, 05.175 and 05.222], an aneugenic potential may also be anticipated. For these four substances, individual data are needed to establish whether they have aneugenic potential. Accordingly, it is currently not appropriate to assess any of these five substances through the Procedure for the evaluation of flavouring substances.

Keywords: FGE.216, α,β-unsaturated 2-phenyl-2-alkenals, flavouring substances, safety evaluation, subgroup 3.3, FGE.19

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1. Introduction

1.1. Background and terms of reference as provided by the requestor

The use of flavourings is regulated under Regulation (EC) No 1334/2008[1] of the European Parliament and Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation an evaluation and approval are required for flavouring substances.

The Union List of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012[2]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[3].

On 4 July 2013 the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids adopted an opinion on Flavouring Group Evaluation 216 Revision 1 (FGE.216 Rev1): Consideration of genotoxicity data on α,β–unsaturated 2-Phenyl-2-Alkenals from Subgroup 3.3 of FGE.19.

The Panel concluded that for representative substance 2-phenylcrotonaldehyde [FL-no: 05.062], the Panel's concern with respect to genotoxicity could not be ruled out and subsequently additional data are requested.

On 7 January 2014 the applicant submitted to the Commission and to EFSA (Ares(2014)96077) additional information on this group of substances. However, this new data does not completely address the request of data by EFSA in its opinion concerning the proof of sufficient systemic exposure to the test substance.

On 15 August 2014 the applicant submitted to the Commission and to EFSA (Ares(2015)886499) analytical data for plasma analysis of the representative substance 2-phenylcrotonaldehyde [FL-no: 05.062].

On 9 January 2015 Ares(2015)202297 the applicant has submitted the final report on the representative substance [FL-no: 05.062]. The study concerns the development and limited validation of a method for the analysis of plasma samples which may contain 2-phenylcrotonaldehyde.

1.1.1. Terms of Reference

The European Commission requests the European Food Safety Authority (EFSA) to evaluate this new information and, depending on the outcome, proceed to the full evaluation of this group of substances (FGE.19 subgroup 3.3) for which the substance [FL-no: 05.062] is representative in accordance with Commission Regulation (EC) No 1565/2000.

2. Data and methodologies

2.1. History of the evaluation of FGE.19 substances

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register being α,β-unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation (EFSA, 2008a).

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity. The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The α,β-unsaturated carbonyls were subdivided into subgroups on the basis of structural similarity (EFSA, 2008a). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure–activity relationship (Q)SAR prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI-MultiCASE Models and ISS-Local Models (Gry et al., 2007)).

[1] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34–50.

[2] Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1–161.

[3] Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, p. 8–16.

The Panel noted that for most of these models, internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these α,β-unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the Procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS local models (Benigni and Netzeva, 2007) and four DTU-NFI MultiCASE models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances.

Based on these data, the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA, 2008a,b) could not be evaluated through the Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 flavouring group evaluations (FGEs) were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

For 11 subgroups, the Panel decided, based on the available genotoxicity data and (Q)SAR predictions, that a further scrutiny of the data should take place before requesting additional data on genotoxicity from the flavouring industry. These subgroups were evaluated in FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. For the substances in FGE.202, 214 and 218, it was concluded that a genotoxic potential could be ruled out, and accordingly, these substances have been evaluated using the Procedure. For all or some of the substances in the remaining FGEs, FGE.201, 203, 210, 212, 213, 216, 217 and 220, the genotoxic potential could not be ruled out.

To ease the data retrieval of the large number of structurally related α,β-unsaturated substances in the different subgroups for which additional data are requested, EFSA has worked out a list of representative substances for each subgroup (EFSA, 2008c). Likewise, an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008b).

The flavouring industry has been requested to submit additional genotoxicity data according to the list of representative substances and test strategy for each subgroup.

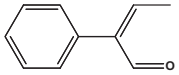
The flavouring industry has submitted additional data and the present revision of FGE.216 (FGE.216Rev2) concerns the evaluation of these data requested on genotoxicity.

2.2. Presentation of the substances in flavouring group evaluation 216

The flavouring group evaluation 216 (FGE.216), corresponding to FGE.19 subgroup 3.3, concerns five α,β-unsaturated 2-phenyl substituted aldehydes, 2-phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222], which are presented in Appendix A, Table A.1. In the EFSA Opinion, 'List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing' (EFSA, 2008c), 2-phenylcrotonaldehyde [FL-no: 05.062] (Table 1) had been selected as representative flavouring substance for FGE.19, subgroup 3.3, corresponding to FGE.216.

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a). Accordingly, the available data on genotoxic or carcinogenic activity for the five aldehydes [FL-no: 05.062, 05.099, 05.100, 05.175 and 05.222] will be considered in this FGE.

Table 1: Representative substance for subgroup 3.3 of FGE.19 (EFSA, 2008c)

FL-no JECFA-no	Subgroup	EU Register name	Structural formula	Comments
05.062 1474	3.3	2-Phenylcrotonaldehyde		Data submitted in accordance to request and evaluated in FGE.216, FGE.216Rev1 and FGE.216Rev2

2.3. History of the evaluation of the substances belonging to FGE.216

In the first scientific opinion on FGE.216 (EFSA, 2009), no data from genotoxicity or carcinogenicity studies with any of the substances in FGE.216 were available. The (Q)SAR predictions of these

substances were limited to one endpoint (gene mutations in one strain of *Salmonella*), see Appendix C, Table C.1. 2-Phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] and 4-methyl-2-phenylpent-2-enal [FL-no: 05.100] were evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) as of no safety concern (JECFA, 2006), see Appendix B, Table B.1.

In FGE.216 (EFSA, 2009), the data available were insufficient to rule out the concern for genotoxicity; therefore, the CEF Panel requested to provide additional data on genotoxicity for the representative substance of this subgroup, according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008b).

The flavouring industry submitted genotoxicity data on the representative substance 2-phenylcrotonaldehyde [FL-no: 05.062] that were evaluated in FGE.216Rev1 (EFSA CEF Panel, 2013): a bacterial reverse mutation assay, an *in vitro* and an *in vivo* micronucleus (MN) assay. The CEF Panel noted that 2-phenylcrotonaldehyde induced micronuclei *in vitro* in the absence of metabolic activation. Equivocal results were obtained in the *in vivo* MN assay with no evidence of bone marrow exposure.

JECFA evaluated the same studies in 2014 (JECFA, 2015) together with a second *in vivo* MN assay, which is evaluated also in the present revision of FGE.216 (FGE.216Rev2). JECFA concluded that the Procedure cannot be applied until concerns regarding genotoxicity are addressed for [FL-no: 05.062, 05.099, 05.100, 05.222].

In FGE.216Rev1 (EFSA CEF Panel, 2013), the CEF Panel requested to provide proof of sufficient systemic exposure of animals treated with 2-phenylcrotonaldehyde [FL-no: 05.062] and to provide an *in vivo* comet assay in the gastrointestinal system.

The flavouring industry has submitted data requested by the CEF Panel in FGE.216Rev1 that are evaluated in the present revision of FGE.216 (FGE.216Rev2).

Sections 2.4 and 2.5 report the same information that was presented in FGE.216 and FGE.216Rev1, respectively. Section 3 presents the evaluation of the new data submitted for 2-phenylcrotonaldehyde [FL-no: 05.062].

FGE	Adopted by EFSA	Link	No. of substances
FGE.216	27 November 2008	https://www.efsa.europa.eu/en/efsajournal/pub/881	5
FGE.216Rev1	4 July 2013	https://www.efsa.europa.eu/en/efsajournal/pub/3305	5
FGE.216Rev2	29 June 2022	https://www.efsa.europa.eu/it/efsajournal/pub/7420	5

2.4. Data evaluated in FGE.216[4]

2.4.1. (Q)SAR predictions

In Appendix C, the outcomes of the (Q)SAR predictions for possible genotoxic activity in five *in vitro* (Q)SAR models (ISS Local Model-Ames test, DTU-NFI MultiCASE-Ames test, -Chromosomal aberration test in Chinese hamster ovary cells (CHO), -Chromosomal aberration test in Chinese hamster lung cells (CHL), and -Mouse lymphoma test) are presented.

For all substances, a negative prediction was obtained in the ISS Local Model for mutagenicity in the Ames test with *Salmonella typhimurium* strain TA100 without activation. For the four DTU-NFI MultiCASE models, these substances were invariably out of domain, meaning that these models did not provide indication on the presence or absence of a genotoxic potential (see Appendix C).

2.4.2. Carcinogenicity studies

No carcinogenicity studies are available for the substances in subgroup 3.3 of FGE.19.

2.4.3. Genotoxicity studies

No genotoxicity studies are available for the substances in subgroup 3.3 of FGE.19.

[4] Data presented in Section 2.4 are cited from the first opinion on FGE.216 (EFSA, 2009).

2.4.4. Conclusion on genotoxicity and carcinogenicity

No data from genotoxicity or carcinogenicity studies with any of the substances in FGE.216 are available. The available (Q)SAR predictions of these substances are limited to one endpoint (gene mutations in one strain of *Salmonella*). The data are insufficient to rule out the concern for genotoxicity.

2.4.5. Conclusions

The Panel concluded that a genotoxic potential of the five 2-phenyl-substituted aldehydes (i.e. 2-phenyl-2-alkenals) in the present FGE.216 could not be ruled out, and therefore, these five substances cannot presently be evaluated through the Procedure. Additional data on genotoxicity for representative substances of this subgroup should be provided, according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008b).

2.5. Additional genotoxicity data considered in FGE.216Rev1[5]

The first revision of FGE.216, Revision 1 (FGE.216Rev1) concerned the evaluation of new genotoxicity data submitted by European Flavour and Fragrance Association (EFFA), in response to the request by EFSA in FGE.216, for the representative substance 2-phenylcrotonaldehyde [FL-no: 05.062], which is supposed to cover the genotoxicity evaluation of the four other substances in FGE.19, subgroup 3.3, 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222].

The new data submitted covered *in vitro* assays in bacteria and mammalian cell systems and *in vivo* data in the rat.

2.5.1. *In vitro* data

2.5.1.1. Bacterial reverse mutation assay

Ames assays were conducted in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 to assess the mutagenicity of 2-phenylcrotonaldehyde [FL-no: 05.062] (98.1% sum of isomers), both in the absence and in the presence of metabolic activation by an Aroclor 1,254-induced rat liver post-mitochondrial fraction (S9-mix) in three separate assays using both standard plate incorporation and modified pre-incubation treatments (Kilford, 2010). The protocol followed OECD Test Guideline (TG) 471 (OECD, 1997a) and the study was performed according to GLP.

In assay 1, no increases in revertant numbers were observed when *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 were incubated with 2-phenylcrotonaldehyde [FL-no: 05.062] up to 5,000 μg/plate in the absence and presence of S9-mix using the standard plate incorporation method. A weak to moderate bacteriostatic activity was noted at concentrations of 1,000 μg/plate and above in strains TA98 and TA102 in the absence of S9-mix and in strains TA1537 and TA102 in the presence of S9-mix.

In assay 2, no increases in revertant numbers were observed when *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 were treated with 2-phenylcrotonaldehyde [FL-no: 05.062] up to 5,000 μg/plate in the absence of S9-mix. In the presence of S9-mix, the same concentrations were tested on strains TA98, TA100 and TA1535, whereas TA1537 and TA102 were treated up to 2000 μg/plate due to an excessive level of cytotoxicity in the first assay. A marked reduction in revertant numbers and/or slight thinning of the bacterial lawn was noted in all the high doses tested. No increase of revertants was observed except in the treatments of the TA100 strain in the absence of S9-mix at a concentration of 2000 μg/plate and in the presence of S9-mix at a concentration of 320 μg/plate. The increase in revertant mutations was statistically significant ($p < 0.01$), but these results were isolated and not reproducible in further assays.

To further explore the increase in mutations seen only in *S. typhimurium* strain TA100, assay 3 was performed in all tester strains in the presence of S9-mix and in the absence of S9-mix in strain TA100. No mutagenic effect was demonstrated.

Under these conditions, 2-phenylcrotonaldehyde [FL-no: 05.062] demonstrated no mutagenic activity in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 both in the absence and in the presence of metabolic activation.

[5] Data presented in Section 2.5 are cited from the first revision of FGE.216 (FGE.216Rev1; EFSA CEF Panel, 2013).

2.5.1.2. Micronucleus assays

2-Phenylcrotonaldehyde [FL-no: 05.062] was tested to determine its clastogenic or aneugenic potential in mammalian cells *in vitro* using the micronucleus test in cultured human peripheral blood lymphocytes with and without metabolic activation (Lloyd, 2012). The test was performed according to OECD TG 487 (OECD, 2010) (except that the assay with metabolic activation was not repeated) and performed according to GLP Guidelines.

The range of concentrations was determined in a preliminary range finding study.

In assay 1, 2-phenylcrotonaldehyde [FL-no: 05.062] was added for 3 h with a 21-h recovery period in the absence of S9-mix and examined at concentrations of 40, 60, 100 and 120 μg/mL. The frequency of micronucleated binucleate (MNBN) cells was statistically higher ($p < 0.001$) than vehicle controls at 100 and 120 μg/mL with 26 and 66% of cytotoxicity, respectively. The frequencies of MNBN cells exceeded the 95th percentile observed range only at 120 μg/mL (in both cultures), indicating a weak but significant induction of chromosomal damage. It was also added to cultures for 3 h with 21-h recovery in the presence of S9-mix at concentrations of 100, 130 and 140 μg/mL. The frequency of MNBN cells was significantly higher ($p < 0.05$) at the two highest concentrations analysed, 130 and 140 μg/mL, but fell clearly within normal ranges based on historical control data. Cultures were also treated for 24 + 0 h in the absence of S9-mix at concentrations of 20, 23 and 26 μg/mL. The frequencies of MNBN cells were significantly higher ($p < 0.05$) than those observed in concurrent vehicle controls at all three concentrations (20, 23 and 26 μg/mL), but also fell within normal ranges based on historical control data. These data were considered difficult to interpret due to the steep concentration related cytotoxicity that was observed under all three treatment conditions as indicated by decreases in the replication index values of 13, 25 and 43%, respectively.

In assay 2, cultures were treated with 2-phenylcrotonaldehyde [FL-no: 05.062] at concentrations of 20, 60, 70 and 80 μg/mL for 3 h with 21-h recovery in the absence of S9-mix. The frequency of MNBN cells was significantly higher ($p < 0.01$) compared to those observed in concurrent vehicle controls at 20, 70 and 80 μg/mL, but not at 60 μg/mL. The MNBN cell frequencies in both cultures at 20 and 70 μg/mL and in one culture at 80 μg/mL exceeded the 95th percentile of the historical control range. These observations indicate the induction of micronuclei at concentrations at or below the limit of cytotoxicity. No second assay was performed with S9-mix.

In conclusion, 2-phenylcrotonaldehyde [FL-no: 05.062] induced a significant increase of micronuclei in cultured human peripheral blood lymphocytes when tested for 3 + 21 h in the absence of rat liver metabolic activation (S9-mix). In the same test system, 2-phenylcrotonaldehyde did not induce micronuclei when tested up to toxic concentrations for 3 + 21 h in the presence of S9-mix and for 24 + 0 h in the absence of S9-mix.

A summary of the *in vitro* data is presented in Appendix D, Table D.1.

2.5.2. *In vivo* data

2.5.2.1. Bone Marrow Micronucleus Induction Assay in the rat

An *in vivo* micronucleus assay in rats was performed in compliance with OECD TG 474 (OECD, 1997b) (Henderson, 2013) to determine whether the results obtained in the initial *in vitro* micronucleus assay reflect the situation *in vivo*.

An initial range-finding study was conducted in Han-Wistar rats to estimate the maximum tolerated dose (MTD) of 2-phenylcrotonaldehyde [FL-no: 05.062] (purity 98%), administered by oral gavage. The dose of 700 mg/kg body weight (bw) per day was selected as the MTD based on displayed toxicity at the higher dose levels.

Groups of six male Han-Wistar rats were treated via gavage with 2-phenylcrotonaldehyde [FL-no: 05.062] at doses of 0 (vehicle control), 70, 350 and 700 mg/kg bw per day. Animals were dosed at 0 and 24 h, followed by sacrifice and harvest of the femoral bone marrow at 24 h after the last treatment.

Rats treated with 2-phenylcrotonaldehyde [FL-no: 05.062] at all doses exhibited group mean % of polychromatic erythrocytes (PCE) that were similar to the vehicle control group. This parameter cannot be used to demonstrate systemic exposure of animals.

In rats treated with 2-phenylcrotonaldehyde [FL-no: 05.062], there were no statistically significant increases in frequency of micronucleated polychromatic erythrocytes (MNPCE) for any of the groups receiving the test article, compared to the concurrent vehicle control, with the exception of the

intermediate dose group, which was nonetheless well within the historical control range and the difference was due to the very low concurrent control frequencies.

The authors of the report concluded that 2-phenylcrotonaldehyde [FL-no: 05.062] did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of male rats treated at 70 and 700 mg/kg bw per day, but that it induced a small statistically significant increase in the frequency of MNPCEs observed at the intermediate dose (350 mg/kg bw per day), and they concluded that the small increase observed at 350 mg/kg bw per day is of questionable biological relevance. The intermediate dose produced a group mean MNPCEs that was twofold greater than and statistically ($p < 0.05$) higher than the vehicle control group. The mean value (2.83 MNPCEs/2000 PCE) was within the laboratory's historical range (0.74–4.46 MNPCEs/2000 PCE). However, individual results of the first reading demonstrated that percent values of MNPCE of 4 out of the 6 treated animals exceed the 95% confidence interval for mean of historical controls and all the individual values of the control animals were within the limit of historical controls.

The data generated from a second set of 2000 PCE gave a similar response across all test article groups with all individual values falling 'normally' within the historical distribution. However, the concurrent vehicle control frequencies were distributed at the low end of the historical data producing a low background level for comparison.

The percent values of MNPCE obtained in the first and second reading are shown in Table 2.

Table 2: *In vivo* bone marrow micronucleus assay (Henderson, 2013), %MNPCE

Treatment	Reading 1	Reading 2	Reading mean
Vehicle	0.10	0.04	0.07
75 mg/kg	0.09	0.12	0.10
350 mg/kg	0.18[(a)]	0.10	0.14[(a)]
700 mg/kg	0.12	0.12	0.12

(a): $p < 0.01$.

The results demonstrate that after a second reading, the increase is still statistically significant when the data from the first and the second reading are pooled.

Plasma of animals of a satellite group was taken, but not analysed for 2-phenylcrotonaldehyde [FL-no: 05.062] content. Under these conditions, no clear proof of exposure was given.

Moreover, 2-phenylcrotonaldehyde [FL-no: 05.062] was found to be positive without metabolic activation in the *in vitro* micronucleus test. After oral absorption, the gastrointestinal tract (GIT) is the organ most exposed to high concentrations. These concentrations will not be achieved in the bone marrow. Therefore, it appears necessary to have information on genotoxicity potential of 2-phenylcrotonaldehyde [FL-no: 05.062] in the gastrointestinal mucosa by a Comet assay in the stomach or duodenum.

A summary of the *in vivo* data are presented in Appendix D, Table D.2.

2.5.3. Conclusion on data considered in FGE.216Rev1

The FGE.216 concerned five α,β-unsaturated 2-phenyl substituted aldehydes, 2-phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222], corresponding to subgroup 3.3 of FGE.19. The conclusion of the CEF Panel in FGE.216 was that the available data on genotoxicity were too limited to evaluate the five substances through the Procedure and additional genotoxicity data were requested.

The flavouring industry has submitted new data in reply to the above requested data for FGE.19 subgroup 3.3 (FGE.216) for the representative flavouring substance, 2-phenylcrotonaldehyde [FL-no: 05.062], covering the remaining four substances [FL-no: 05.099, 05.100, 05.175 and 05.222].

2-Phenylcrotonaldehyde [FL-no: 05.062] did not demonstrate any mutagenic effect in a bacterial test with and without metabolic activation. However, it showed a genotoxic effect in the *in vitro* micronucleus test in cultured human lymphocytes in the absence of metabolic activation.

In order to verify that this genotoxic potential demonstrated *in vitro* was confirmed *in vivo*, a micronucleus test was conducted in the rat bone marrow by oral route which led to an ambiguous result, because only the intermediate dose induced a statistically significant increase of MNPCE, even after rereading the slides. No evidence of systemic exposure of animals was provided in this study, in

particular, no change in the percentage of PCE in the bone marrow was noted and plasma of animals sampled in a satellite group have not been analysed.

Under these conditions, it appears necessary to provide proof of sufficient systemic exposure of animals treated with 2-phenylcrotonaldehyde.

Moreover, since the substance was genotoxic only without metabolic activation, it appears necessary to prove the absence of genotoxic effect locally in the gastrointestinal system using the Comet assay.

3. Assessment

3.1. Additional data considered by the Panel in FGE.216Rev2

Data on genotoxicity

Following the request for additional data expressed by the CEF Panel in FGE.216Rev1, the industry has investigated the presence of 2-phenylcrotonaldehyde [FL-no: 05.062] in the plasma of a satellite group of animals (Covance, 2014) from two *in vivo* MN assays by Henderson (2013) and Covance (2013). The industry has repeated the *in vivo* MN assay using the same experimental conditions described in the study by Henderson, 2013 (Covance, 2013).

Upon request by EFSA (EFSA letter sent on 19 May 2015), an *in vivo* comet assay with scoring of duodenum cells has been submitted (Covance, 2016) on 1 July 2016. Since the available data did not allow to evaluate the aneugenic potential of 2-phenylcrotonaldehyde [FL-no: 05.062], the CEF Panel requested (EFSA letter sent on 9 February 2017) to test this substance in an *in vitro* MN assay with centromere staining. On 12 February 2018, the applicant provided the study requested (BioReliance, 2018a) and additional studies: a bacterial reverse mutation assay (BioReliance, 2016) and an *in vivo* gene mutation assay in transgenic mice (BioReliance, 2017, 2018b) that are evaluated in the present revision of FGE.216 (FGE.216Rev2). The new data evaluated in FGE.216Rev2 are listed in Table 3.

Table 3: Data evaluated in FGE.216Rev2

Chemical name [FL-no]	Additional data submitted	Reference
2-Phenylcrotonaldehyde [FL-no: 05.062]	*In vitro* bacterial reverse mutation assay	BioReliance (2016)
	In vitro MN assay with CREST staining in TK6 cells	BioReliance (2018a)
	Development and limited validation of a method for the analysis of plasma samples which may contain 2-phenylcrotonaldehyde	Covance (2014)
	In vivo MN assay in bone marrow	Covance (2013)
	In vivo comet assay in duodenum	Covance (2016)
	In vivo Oral Dose Range Finding Assay in C57BL/6 Mice	BioReliance (2017)
	In vivo Mutation Assay at the cII Locus in Big Blue® Transgenic C57BL/6 Mice	BioReliance (2018b)
	Use levels	Documentation provided to EFSA No. 10
5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175], 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222]	Use levels	Documentation provided to EFSA No. 10

Data on uses and use levels and natural occurrence

New data on uses and use levels have been provided, documentation provided to EFSA No. 9 and No. 10, following a request by EFSA sent on 16 May 2018 and 8 November 2021, respectively. This latest submission (documentation provided to EFSA No. 10) replaces uses and use levels data provided

in 2018 (documentation provided to EFSA No. 9). These data have been used in Appendix F to estimate exposure.

Based on information retrieved by EFSA from the Volatile Compounds in Food (VCF) database (VCF online database, 2022), the substances [FL-no: 05.062, 05.099, 05.100, 05.175] are reported to be present in unprocessed and processed food. For substance [FL-no: 05.222], no occurrence data are reported (Appendix F).

3.2. *In vitro* data

3.2.1. Bacterial reverse mutation assay

2-Phenylcrotonaldehyde (purity: 97.6% sum of isomers, as per 'post-study' final analytical report) was tested for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2 uvrA in the presence or absence of S9-mix (prepared from Aroclor-induced rats) (BioReliance, 2016). The study was performed according to GLP and OECD TG 471 (OECD, 1997a).

A preliminary toxicity-mutation assay was performed to determine the range of concentrations to be used in the confirmatory assay. 2-Phenylcrotonaldehyde was tested at 1.5, 5.0, 15, 50, 150, 500, 1,500 and 5,000 μg/plate in all strains both with and without S9-mix (two plates per concentration). DMSO was used as vehicle control.

No precipitate was observed. Toxicity was observed at the two highest concentrations (1,500 or at 5000 μg/plate). An increase in the frequency of revertants was observed in *E. coli* WP2 uvrA in the presence and absence of S9-mix. Increases were observed also in *S. typhimurium* strains TA98 and TA100 in the absence of S9-mix and in strain TA1535 both in the presence and absence of S9-mix. These increases in revertant counts were not clearly concentration-related or consistently outside the historical control limits; therefore, in the confirmatory assay, the range of concentrations tested was adjusted in order to clarify the responses observed.

In the confirmatory assay, 2-phenylcrotonaldehyde was tested at 15, 50, 150, 500, 1,000, 1,500 and 5,000 μg/plate with the plate incorporation method (in triplicate). No precipitate was observed. Toxicity was observed at 1,500 or at 5,000 μg/plate. Increases in revertant counts were observed in *S. typhimurium* strains TA98 and TA100 without metabolic activation and with tester strains TA100 in the presence of metabolic activation (1.5-,1.9-, 1.6-fold, respectively). The authors of the study noted that some of the counts are outside of the historical control limits, but none of these increases were concentration-related.

Increases in the frequency of revertants were confirmed in *S. typhimurium* strain TA1535 and in *E. coli* WP2 uvrA in the presence (2.8-, 11.7-fold, respectively) and absence of S9-mix (6.3-, 19.6-fold respectively).

No increase was observed with tester strain TA98 in the presence of S9-mix. Due to tester strain contamination, tester strain TA1537 was not evaluated for mutagenicity but was retested in a further experiment with the same concentrations of 2-phenylcrotonaldehyde.

In this experiment, no increases in the frequency of revertants were observed in *S. typhimurium* strain TA1537 in either the presence or absence of S9-mix. No precipitate was observed. Toxicity was observed at the highest concentrations tested (1,500 or at 5,000 μg/plate).

The authors of this study considered as equivocal the increase of revertants observed in strain TA100 in the absence of S9-mix and in strain TA1535 in the presence of S9-mix. The increase in revertants observed in strain TA98, in the absence of metabolic activation, was not concentration-related and was inside the range of historical controls. The authors considered the results observed in TA98 as negative. They concluded that 2-phenylcrotonaldehyde is positive in *S. typhimurium* strain TA1535, without S9-mix, and in *E. coli* WP2 uvrA in the presence and absence of S9-mix.

The Panel concluded that 2-phenylcrotonaldehyde is positive in *S. typhimurium* strain TA1535, without S9-mix, and in *E. coli* WP2 uvrA in the presence and absence of S9-mix and equivocal in strain TA100 in the absence of S9-mix and in strain TA1535 in the presence of S9-mix.

3.2.2. *In vitro* micronucleus assay with kinetochores staining

2-Phenylcrotonaldehyde (purity 99%) was tested in an *in vitro* micronucleus assay in the human lymphoblastoid cell line TK6 cells (BioReliance, 2018a), with the purpose of evaluating the aneugenic and clastogenic potential of the tested substance. DMSO was used as the vehicle. The study was performed according to GLP and OECD TG 487 (OECD, 2014a).

TK6 cells were treated for 4 h with 23 h of recovery period (4 + 23 h) in the absence or presence of S9-mix (from aroclor-induced rats) or for 27 h in the absence of S9-mix. Positive controls were: mytomycin C (MMC), cyclophosphamide (CPA), vinblastine (VB).

In the preliminary toxicity test, concentrations between 0.146 and 1,460 μg/mL were tested. Precipitate was observed at concentrations above 438 μg/mL for all the three treatment conditions.

Cytotoxicity (more than 50% decrease in relative population doubling (RPD) compared to the vehicle control) was observed at concentrations above 146 μg/mL for the 4 h treatment both in the absence and in the presence of S9-mix, and at concentrations above 43.8 μg/mL for the 27-h treatment without S9-mix.

Based on the results of the preliminary toxicity test, the following concentrations were tested:

- For the treatment 4 + 23 h in the absence of metabolic activation 5, 10, 20, 40, 45, 50 μg/mL;
- For the treatment 4 + 23 h in the presence of metabolic activation 20, 40, 60, 80, 100, 120, 140 μg/mL;
- For the treatment at 27 h in the absence of metabolic activation 5, 15, 20, 25, 30, 35, 40 μg/mL;

Each concentration was tested in duplicate cultures; for each culture, 1,000 mononucleated cells were analysed for MN (a total of 2,000 cells per concentration). Due to cytotoxicity of approximatively 50%, the highest concentrations evaluated for MN induction were 45 μg/mL (for the 4 + 23 h treatment without S9-mix), 20 μg/mL (for the 27 h treatment without S9-mix) and 80 μg/mL (for the 4 + 23 h treatment with S9-mix).

After 4 h treatment +23 h recovery period, in the absence of S9-mix, at 20, 40 and 45 μg/mL and in the presence of S9-mix, at 40, 60 and 80 μg/mL, no increase in micronucleated cell frequency was observed.

After 27 h treatment without recovery period, in the absence of S9-mix, a statistically significant and concentration-dependent increase in micronucleated cell frequency was observed at 20 μg/mL.

In order to confirm this positive result, the micronucleus assay was repeated with a narrower range of concentrations: 5, 16, 18, 20, 22, 24, 26 μg/mL. The cytotoxicity observed at the highest concentration tested was 41%; therefore, the experiment was repeated again at the following concentrations: 5, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 45 μg/mL. In this third experiment, a cytotoxicity of 57% was observed at the concentration tested of 20 μg/mL, that was the highest concentration analysed for MN induction. No significant increase in micronucleated cell frequency was observed at 16 and 18 μg/mL, but a statistically significant increase in MN induction (1.25%) was observed at 20 μg/mL ($p \leq 0.01$) versus 0.55% in the negative control. However, the effect was not concentration-related (the Cochran–Armitage test was statistically not significant for a concentration-dependent response ($p > 0.05$)).

The micronuclei induced by 2-phenylcrotonaldehyde 20 μg/mL (after 27 h of treatment) were analysed through CREST staining (antikinetochore antibody staining) in order to determine the mechanism of action (aneugenicity or clastogenicity). The same was applied to vehicle and positive controls.

The results of the CREST analysis indicate that 77% of MN induced by 2-phenylcrotonaldehyde were positive for kinetochore staining. The clastogen-positive control MMC showed 30% of MN positive for kinetochore staining. The negative control showed 60% of MN positive for kinetochore staining. The aneugen-positive control VB had 87% of MN positive for kinetochore staining. Therefore, the authors of this study considered that 2-phenylcrotonaldehyde induces micronuclei in TK6 cells after 27 h of treatment, without metabolic activation, via an aneugenic mechanism.

The Panel agrees with this conclusion.

Results of *in vitro* studies are summarised in Appendix E, Table E.1.

3.3. *In vivo* data

3.3.1. Plasma bioanalysis

In order to demonstrate the bone marrow exposure of animals treated with 2-phenylcrotonaldehyde [FL-no: 05.062] in the micronucleus assay by Henderson, 2013 (see Section 2.5.2.1) and by Covance, 2013 (see Section 3.3.2), a plasma analysis of a satellite group of animals was provided (Covance, 2014). Six male Han Wistar rats were dosed, by oral gavage, with 700 mg/kg bw per day (an estimate of the MTD) of 2-phenylcrotonaldehyde. A method has been developed for the analysis of 2-phenylcrotonaldehyde using gas chromatography with mass selective

detection (GC–MSD). The method was developed from a literature method using a gas chromatography methodology with the mass selective detector in scan mode, and suitable ions were selected for use in single ion monitoring mode. A procedure was also developed to extract 2-phenylcrotonaldehyde from the rat plasma samples.

According to the study authors, satisfactory linearity, recovery and repeatability were found for 2-phenylcrotonaldehyde when the substance was spiked and analysed in rat plasma samples.

The Panel, however, noted that linearity in plasma extracts was in the range of 10–400 μg/mL, but the highest concentration reported for 2-phenylcrotonaldehyde in rat plasma samples was below this range. Moreover, the recovery and accuracy of the method were only determined from 50 μg/mL and above.

No animals in the vehicle control group showed any detectable levels of 2-phenylcrotonaldehyde in plasma, while less than 10 μg/mL 2-phenylcrotonaldehyde were detected in the plasma of animals treated with 700 mg/kg bw per day, half an hour after dosage. The Panel, however, noted that there is some variability among treated animals. Although these results indicate that a very low amount of 2-phenylcrotonaldehyde might be present in plasma shortly after dosing, the Panel concluded that the validation package provided by the study is not robust enough on its own to support this conclusion.

3.3.2. *In vivo* micronucleus assay

2-Phenylcrotonaldehyde [FL-no: 05.062] (purity 99.66%) was tested in a second bone marrow MN assay in rats which was performed in compliance with GLP and according to OECD TG 474 (OECD, 1997b) (Covance, 2013) as follow-up of the *in vivo* MN assay by Henderson (2013). Based on the dose range finding study already performed by Henderson (2013), the same doses of 2-phenylcrotonaldehyde [FL-no: 05.062] were used in the new study by Covance (2013).

Groups of six Han-Wistar rats were treated via gavage with 2-phenylcrotonaldehyde [FL-no: 05.062] at doses of 0 (vehicle control, aqueous methylcellulose), 70, 350 and 700 mg/kg bw per day. Cyclophosphamide at the concentration of 20 mg/kg bw per day was used as positive control. Animals were dosed at 0 and 24 h, except the positive control group that was dosed only at 24 h. All animals were sacrificed at 48 h and femoral bone marrow was harvested and prepared for the MN analysis.

The vehicle control data were comparable with the laboratory's historical vehicle control data. Positive controls resulted in a statistically significant increase in MNPCE (over the concurrent vehicle control), which was comparable with the laboratory's historical positive control data.

In rats treated with 2-phenylcrotonaldehyde [FL-no: 05.062], in all the three dose groups, there were no statistically significant increases in MNPCE frequency compared to the vehicle controls. Individual frequencies of MNPCE for all treated animals were consistent with historical vehicle control data.

2-Phenylcrotonaldehyde [FL-no: 05.062] did not induce micronuclei in PCE of male rats treated up to 700 mg/kg bw per day (an estimate of the MTD for this study). The PCE/NCE ratio was not affected by the treatment with 2-phenylcrotonaldehyde [FL-no: 05.062], indicating that the treatment did not induce bone marrow toxicity. Since signs of toxicity were not observed up to the highest dose tested (700 mg/kg bw per day), there is no evidence to presume systemic exposure to 2-phenylcrotonaldehyde [FL-no: 05.062]. Also taking into account the plasma analysis data (see above), the Panel considered that no clear evidence of bone marrow exposure is available. Therefore, the Panel evaluated the results of this *in vivo* MN assay as inconclusive.

3.3.3. *In vivo* comet assay

2-Phenylcrotonaldehyde (purity 98.42%) was tested for its potential to induce DNA damage in the duodenum of treated rats (Covance, 2016). The study was performed according to GLP and OECD TG 489 (OECD, 2014b).

Six male Han Wistar rats were dosed twice with 2-phenylcrotonaldehyde at 0 (Day 1) and 21 h (Day 2) by gavage. Dose levels were 175, 350 or 700 mg/kg bw per day. The maximum dose (700 mg/kg bw per day) was an estimate of the MTD based on toxicity data from a previous rat MN study by Henderson (2013). The vehicle was 0.5% (w/v) aqueous methylcellulose and the positive control was ethyl methanesulfonate (150 mg/kg bw per day, single oral administration at 21 h (Day 2)). No signs of clinical toxicity were observed.

Liver and duodenum were sampled on Day 2, 24 h after the first dosing. Only duodenum was analysed for comet assay.

There were no clinical chemistry findings considered to be related to administration of 2-phenylcrotonaldehyde. Histopathology did not reveal any macroscopic or microscopic findings associated with the administration of 2-phenylcrotonaldehyde.

The vehicle control data were comparable with the laboratory's historical data. The positive control resulted in a statistically significant increase in tail intensity (over the concurrent vehicle control) that was comparable with the laboratory's historical positive control data.

There was no dose-related increase in % hedgehogs in duodenum following treatment with 2-phenylcrotonaldehydethus demonstrating that the treatment did not cause excessive DNA damage, which could interfere with comet analysis.

No statistically significant increases in tail intensity at any dose levels of 2-phenylcrotonaldehyde was observed compared to the vehicle control. All individual animal data at all dose levels were consistent with the data from vehicle control animals and fell within the laboratory's historical control data. The Panel concluded that 2-phenylcrotonaldehyde did not induce DNA damage in the duodenum of rats treated up to 700 mg/kg bw per day (estimated MTD).

3.3.4. *In vivo* gene mutation assay in Big Blue® transgenic mice

2-Phenylcrotonaldehyde was tested in a 5-day dose range finding assay in C57BL/6 mice (BioReliance, 2017), in order to determine the MTD. The study was performed in compliance with GLP. Results from this study were used for selecting the dose levels tested in the *in vivo* gene mutation assay in Big Blue® Transgenic C57BL/6 mice (BioReliance, 2018b).

2-Phenylcrotonaldehyde (purity 99%) was administered via oral gavage (0.5% methylcellulose used as vehicle) to five groups of wild-type C57BL/6 male mice (three animals per group) corresponding to different dose levels: 50, 100, 250, 500, 1,000 mg/kg bw per day. An additional group (Group 6) of three Big Blue® C57BL/6 homozygous transgenic male mice was dosed at 750 mg/kg bw per day in order to verify whether the top dose selected for the 28-day dosing period was the MTD.

All animals were observed twice daily, at least 6 h apart, for moribundity and mortality. Clinical observations were performed pre-dose on Day 1 (Groups 1–6) and prior to sacrifice on Day 5 (Groups 1–5 only).

The three animals dosed at 1,000 mg/kg bw per day were found death or sacrificed moribund on day 1, 3 and 4. Prostration, laboured breathing, cold to touch and\or decreased motor activity was documented in all three animals treated with 1,000 mg/kg bw per day. One of the three transgenic mice dosed with 750 mg/kg bw was sacrificed in moribund condition a few hours after the first dose due to prostration and decreased motor activity.

No body weight changes associated with 2-phenylcrotonaldehyde treatment were observed at any dose level up to 5 days. Based on these results, the authors of this study recommended for the 28-day study in transgenic mice the doses of 100, 300 and 670 mg/kg bw per day (BioReliance, 2017). Upon further review of the available toxicity data, the dose of 670 mg/kg bw per day was considered to potentially exceed the MTD over the 28-day treatment period. Therefore, for the *in vivo* gene mutation assay, the dose of 500 mg/kg bw per day was chosen as the highest dose because no mortality or toxicity were observed. The lower doses selected were 75 and 250 mg/kg bw per day.

In the *in vivo* gene mutation assay (BioReliance, 2018b), 2-phenylcrotonaldehyde (purity 99%) was administered via oral gavage (0.5% methylcellulose used as vehicle) to four groups of male transgenic Big Blue® C57BL/6 mice (6 animals per group) at the dose levels of 0, 75, 250 and 500 mg/kg bw per day. This study was performed according to GLP and OECD TG 488 (OECD, 2013).

Dose formulations were prepared fresh each day just prior to dosing.

One animal treated at 500 mg/kg bw per day was terminated in moribund condition on Day 12. Ataxia, decreased motor activity, prostration, laboured breathing, white discharge from the eyes and squinting were noted at 500 mg/kg bw per day in all animals, mainly during the first week of treatment. Decreased motor activity was also noted in one animal at 250 mg/kg bw per day on Day 9.

Total absolute body weight gains were lower than control for all test article treated groups, reaching statistical significance at the 75 and 500 mg/kg bw per day. No differences in food consumption were observed.

Liver, duodenum and bone marrow samples from at least five animals per group were processed for DNA isolation. In addition, frozen tissue samples for liver, duodenum and bone marrow were processed for DNA isolation from five positive control animals, previously exposed to ENU (N-ethyl-N-nitrosurea, via oral gavage, 40 mg/kg bw per day on Days 1, 2 and 3) in another study of the same laboratory.

Sufficient quantity and quality of DNA was obtained to permit 2–6 packagings of each DNA sample, yielding more than the OECD-specified minimum of 125,000 plaques per tissue per animal.

Treatment with 2-phenylcrotonaldehyde did not increase the mutation frequency at the cII gene in liver, bone marrow and duodenum of Big Blue® mice.

The authors concluded that in this *in vivo* gene mutation assay in transgenic mice, 2-phenylcrotonaldehyde induced dose-related toxicity based on: one early death, signs of toxicity at 500 mg/kg bw per day and a trend towards lower weekly body weights and total weight gain in all three treatment groups compared to the vehicle control. 2-Phenylcrotonaldehyde did not induce a statistically significant increase in mutation frequency in duodenum, liver and bone marrow.

The Panel agrees with these conclusions.

Results of *in vivo* studies are summarised in Appendix E, Table E.2.

4. Discussion

2-Phenylcrotonaldehyde [FL-no: 05.062] was negative in a bacterial gene mutation assay (Kilford, 2010) with and without metabolic activation. In a second study by BioReliance (2016), a statistically significant increase in revertants was observed in *S. typhimurium* strain TA1535, without S9-mix, and in *E. coli* WP2 uvrA in the presence and absence of S9-mix.

In an *in vivo* follow-up study, 2-phenylcrotonaldehyde was tested for gene mutations in Big Blue® transgenic mice in which duodenum, liver and bone marrow were analysed. No statistically significant increase in mutation frequency was observed in the tissues analysed. The Panel considered that 2-phenylcrotonaldehyde did not induce gene mutations in this *in vivo* assay.

Positive results were observed in two *in vitro* MN assays, in human peripheral blood lymphocytes and in TK6 cells in the absence of metabolic activation.

In the *in vitro* MN assay in TK6 cells, a statistically significant increase in micronucleated cell frequency was observed in the long-term treatment, at a concentration of 20 μg/mL, in the absence of metabolic activation. The CREST analysis showed that 2-phenylcrotonaldehyde [FL-no: 05.062] induced MN mainly through an aneugenic mechanism.

As *in vivo* follow-up study, a first rat bone marrow MN assay was performed by gavage (Henderson, 2013). The CEF Panel considered the results of this study as inconclusive because only the intermediate dose induced a statistically significant increase of MNPCE and there was no evidence of systemic exposure. In a second rat bone marrow micronucleus assay, performed under the same conditions, no increase in MNPCE was observed (Covance, 2013), but also in this study, there was no evidence of bone marrow exposure. Moreover, the results of a separate study on plasma bioanalysis (Covance, 2014) in rats treated with 2-phenylcrotonaldehyde (700 mg/kg bw per day) did not provide a clear evidence of bone marrow exposure, either.

Since signs of toxicity were not observed up to the highest dose tested (700 mg/kg bw per day), there is no evidence of systemic exposure to 2-phenylcrotonaldehyde [FL-no: 05.062]. Therefore, the Panel concluded that results from the two *in vivo* MN studies are inconclusive.

Since 2-phenylcrotonaldehyde was found to be positive without metabolic activation in the *in vitro* micronucleus test, the Panel agreed that *in vivo* the GIT would be the organ most exposed to high concentrations of the substance after oral exposure.

The negative results observed in the *in vivo* comet assay (Covance, 2016) in duodenum can overrule the potential clastogenicity at the site of contact, but not a possible aneugenic effect.

The Panel noted that at present, there are no validated *in vivo* methods to investigate aneugenicity in the GIT.

Following the recommendations for risk assessment in the EFSA Scientific Committee guidance on aneugenicity (EFSA Scientific Committee, 2021), the Panel compared the concentration resulting in aneugenicity *in vitro* (20 μg/mL) with the estimated concentration of the substance in the GIT following ingestion of food or beverage. Since the dilution in the upper parts of the GIT can be considered to be small, the estimated concentration of a substance in this part of the GIT would be in the same order of magnitude as its concentration in food and beverages.

The Panel noted that for 2-phenylcrotonaldehyde [FL-no: 05.062], for some food categories, the normal use levels and/or the maximum use levels (see Appendix F) are less than one order of magnitude below the concentration for which an aneugenic effect of this flavouring substance was observed in the *in vitro* MN assay (i.e. 20 μg/mL). Therefore, for the application in the respective food categories, the use of the flavouring substance [FL-no: 05.062] at the reported use levels would raise a concern for aneugenicity.

Based on structural similarity, for the remaining four substances in this FGE [FL-no: 05.099, 05.100, 05.175 and 05.222], an aneugenic potential may also be anticipated. However, the lowest concentration resulting in aneugenicity *in vitro* for [FL-no: 05.062] cannot be used for the safety assessment for these four substances, because quantitative extrapolation of this concentration to estimate the aneugenic potency of these four substances would be connected to a high level of uncertainty. Therefore, for these four substances, individual data are needed to establish whether they have aneugenic potential. These four substances should be tested in an *in vitro* MN test with centromeres analysis. In case of increase in micronucleated cells frequency, an appropriate follow-up test should be done, according to the EFSA guidance on genotoxicity testing strategy (EFSA Scientific Committee, 2011) and the EFSA guidance on aneugenicity (EFSA Scientific Committee, 2021).

5. Conclusions

The Panel concluded that, for all the substances in this FGE, concern for gene mutation and clastogenicity is ruled out by the negative results observed in the *in vivo* gene mutation assay and in the *in vivo* comet assay, respectively.

2-Phenylcrotonaldehyde [FL-no: 05.062] induced MN *in vitro* through an aneugenic mode of action. The available *in vivo* MN studies were inconclusive and cannot be used to rule out the potential aneugenicity of [FL-no: 05.062] *in vivo*. Therefore, the Panel compared the lowest concentration resulting in aneugenicity *in vitro* with the use levels reported for this substance. Based on this comparison, the Panel concluded that the use of the flavouring substance [FL-no: 05.062] at the reported use levels in several food categories would raise a concern for aneugenicity.

Based on structural similarity, for the remaining four substances in this FGE [FL-no: 05.099, 05.100, 05.175 and 05.222], an aneugenic potential may also be anticipated. However, the lowest concentration resulting in aneugenicity *in vitro* for [FL-no: 05.062] cannot be used for the safety assessment of these four substances, because quantitative extrapolation of this concentration to estimate the aneugenic potency of these four substances would be connected to a high level of uncertainty. Therefore, for these four substances, individual data are needed to establish whether they have aneugenic potential.

Accordingly, it is currently not appropriate to assess any of these five substances through the Procedure for the evaluation of flavouring substances.

6. Documentation as provided to EFSA

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Abbreviations

BW	Body Weight
CAS	Chemical Abstract Service
CEF	Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHO	Chinese hamster ovary (cells)
CHL	Chinese hamster lung (cells)
CoE	Council of Europe
FAO	Food and Agriculture Organisation of the United Nations
FEMA	Flavour and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS	Flavour Information System database
GLP	Good Laboratory Practice
ID	Identity
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MN	Micronucleus
MNBN	MicroNucleated BiNucleate cells
MNPCE	MicroNucleated PolyChromatic Erythrocytes
MS	Mass Spectrometry
MTD	Maximum Tolerated Dose
NMR	Nuclear Magnetic Resonance
No	Number
OECD	Organisation for Economic Co-operation and Development
PCE	PolyChromatic Erythrocytes
(Q)SAR	(Quantitative) Structure–Activity Relationship
WHO	World Health Organisation

Appendix A – Specification Summary of the Substances in the Flavouring Group Evaluation 216Rev2

Table A.1: Specification Summary of the Substances in the Flavouring Group Evaluation 216Rev2 (JECFA, 2006)

FL-no JECFA-no	Chemical name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility[(a)] Solubility in ethanol[(b)]	Boiling point, °C[(c)] Melting point, °C ID test Assay minimum	Refrac. Index[(d)] Spec. gravity[(e)]
05.062 1474	2-Phenylcrotonaldehyde		3224 670 4411-89-6	Liquid $C_{10}H_{10}O$ 146.19	Insoluble Soluble	177 (20 hPa) NMR 97%	1.558–1.564 1.031–1.037
05.099 1472	5-Methyl-2-phenylhex-2-enal		3199 10365 21834–92-4	Liquid $C_{13}H_{16}O$ 188.27	Insoluble Soluble	96–100 (0.9 hPa) NMR 96%	1.531–1.536 0.970–0.976
05.100 1473	4-Methyl-2-phenylpent-2-enal		3200 10366 26643–91-4	Liquid $C_{12}H_{14}O$ 174.24	Insoluble Soluble	96 (0.9 hPa) NMR 95%	1.533–1.539 0.980–0.986
05.175	2-Phenylpent-2-enal		3491-63-2	Liquid $C_{11}H_{12}O$ 160.22	Practically insoluble or insoluble Freely soluble	126 (15 hPa) MS 95%	1.545–1.553 1.005–1.015
05.222 2069	2-Phenyl-4-methyl-2-hexenal		4194 26643–92-5	Liquid $C_{13}H_{16}O$ 188.27	Insoluble Soluble	97 (0.6 hPa) 95%	1.522–1.530 0.965–0.975

(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95% ethanol, if not otherwise stated.
(c): At 1,013.25 hPa, if not otherwise stated.
(d): At 20°C, if not otherwise stated.
(e): At 25°C, if not otherwise stated.

Appendix B – Summary of Safety Evaluation

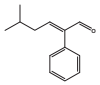
Table B.1: Summary of Safety Evaluation by JECFA of the substances in FGE.19 subgroup 3.3, applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 2006, 2015)

FL-no JECFA-no CAS no	EU Register name	Structural formula	MSDI[(a)] (μg/capita per day)	Class[(b)] Evaluation procedure path[(c)] (JECFA)	Outcome on the named compound (JECFA, 2006)	Outcome on the named compound (JECFA, 2015)	EFSA conclusion on the named compound (genotoxicity)
05.062 1474 4411-89-6	2-Phenylcrotonaldehyde		1.7	Class I A3: Intake below threshold	(d)	(e)	Concern for aneugenicity cannot be ruled out at the reported use levels.
05.099 1472 21834–92-4	5-Methyl-2-phenylhex-2-enal		15	Class II A3: Intake below threshold	(d)	(e)	Concern for aneugenicity cannot be ruled out. Additional genotoxicity data required.
05.100 1473 26643–91-4	4-Methyl-2-phenylpent-2-enal		0.34	Class II A3: Intake below threshold	(d)	(e)	Concern for aneugenicity cannot be ruled out. Additional genotoxicity data required.
05.175 – 3491-63-2	2-Phenylpent-2-enal		0.011	No evaluation	Not evaluated by JECFA	Not evaluated by JECFA	Concern for aneugenicity cannot be ruled out. Additional genotoxicity data required.
05.222 2069 26643–92-5	2-Phenyl-4-methyl-2-hexenal		3.0	No evaluation	Not evaluated by JECFA	(e)	Concern for aneugenicity cannot be ruled out. Additional genotoxicity data required.

(a): EU MSDI: Amount added to food as flavour in (kg/year) × $10E^9$/(0.1 × population in Europe (= 375 × $10E^6$) × 0.6 × 365) = μg/capita per day, calculated by EFSA based on the most recent data submitted.
(b): Thresholds of toxicological concern: Class I = 1,800 μg/person per day, Class II = 540 μg/person per day, Class III = 90 μg/person per day.
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
(d): No safety concern based on intake calculated by the MSDI approach of the named compound.
(e): The Procedure cannot be applied until concerns regarding genotoxicity of 2-phenyl-2-butenal (JECFA No. 1474) are resolved and the evaluation should be reconsidered at a future meeting.

Appendix C – (Q)SAR Predictions on Mutagenicity in Five Models for Five Aldehydes from Subgroup 3.3

Table C.1: (Q)SAR Predictions on Mutagenicity for Five Aldehydes from Subgroup 3.3 considered in FGE.216 (EFSA, 2009)

FL-no JECFA-no	Chemical name	Structural formula[a]	FEMA no CoE no CAS no	ISS Local Model Ames Test TA100[b]	MultiCASE Ames test[c]	MultiCASE Mouse lymphoma test[d]	MultiCASE Chromosomal aberration test in CHO[e]	MultiCASE Chromosomal aberration test in CHL[f]
05.062 1474	2-Phenylcrotonaldehyde		3224 670 4411-89-6	NEG	OD	OD	OD	OD
05.099 1472	5-Methyl-2-phenylhex-2-enal		3199 10365 21834–92-4	NEG	OD	OD	OD	OD
05.100 1473	4-Methyl-2-phenylpent-2-enal		3200 10366 26643–91-4	NEG	OD	OD	OD	OD
05.175	2-Phenylpent-2-enal		– – 3491-63-2	NEG	OD	OD	OD	OD
05.222 2069	2-Phenyl-4-methyl-2-hexenal		– – 26643–92-5	NEG	OD	OD	OD	OD

(Q)SAR: (Quantitative) Structure–Activity Relationship; FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); JECFA: The Joint FAO/WHO Expert Committee on Food Additives; FEMA: Flavour and Extract Manufacturers Association; CoE: Council of Europe; CAS: Chemical Abstract Service; CHO: Chinese hamster ovary (cells); CHL: Chinese hamster lung (cells); NEG: Negative; OD: Out of applicability domain (not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physico-chemical, structural, biological, etc.)

(a): Structure group 3.3: α, β-unsaturated 2-phenyl substituted aldehydes.
(b): Local model on aldehydes and ketones, Ames TA100.
(c): MultiCase Ames test.
(d): MultiCase Mouse Lymphoma test.
(e): MultiCase Chromosomal aberration in CHO.
(f): MultiCase Chromosomal aberration in CHL.

Appendix D – Genotoxicity Data on 2-phenylcrotonaldehyde [FL-no: 05.062] evaluated in FGE.216Rev1

Table D.1: Summary of *in vitro* genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] of subgroup 3.3

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
2-Phenylcrotonaldehyde [FL-no: 05.062]	Reverse Mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	1.6, 8, 40, 200, 1,000 and 5,000 µg/plate[(a),(b)]	Negative	Kilford (2010)	Toxicity was observed in all strains at 5,000 µg/plate in the absence and presence of S9-mix, and at 1,000 µg/plate and above in strains TA98 and TA102 in the absence of S9-mix and in strains TA1537 and TA102 in the presence of S9-mix. All strains were negative. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA98, TA100, TA1535 TA1537 and TA102	51.2, 128, 320, 800, 2,000 and 5,000 µg/plate[(b),(d)]	Negative	Kilford (2010)	Toxicity was observed in strains TA1537 and TA102 at 2,000 µg/plate and above in the absence of S9-mix and at 320 µg/plate in the presence of S9-mix. Similar toxicity was also observed in strains TA98, TA100 and TA1535 at 5,000 µg/plate in the absence of S9 and at 800 µg/plate and above in the presence of S9-mix. Statistically significant differences in mutation frequency were observed only in strain TA100 and only at levels of toxicity (in the absence of S9-mix at a concentration of 2,000 µg/plate and in the presence of S9-mix at 320 µg/plate). Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA98, TA100, TA1535	51.2, 128, 320, 800, 2,000 and 5,000 µg/plate[(c),(e)]	Negative		
		S. typhimurium TA1537 and TA102	20.48, 51.2, 128, 320, 800, 2,000 µg/plate[(c),(e)]	Negative		
		S. typhimurium TA98, TA100, TA1535	31.25–1,000 µg/plate[(c),(e)]	Negative	Kilford (2010)	Toxicity was observed at 3,500 µg/plate and above in strain TA100 in the absence of S9-mix. In the presence of S9-mix, toxicity was observed at 250 µg/plate and above in strains TA1537 and TA102 and at 1,000 µg/plate and above in strains TA100, TA98 and TA1535.
		S. typhimurium TA1537, TA102	15.625–500 µg/plate[(c),(e)]	Negative		
		S. typhimurium TA100	320–5,000 µg/plate[(b),(d)]	Negative		

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
	Micronucleus induction	Human peripheral blood lymphocytes	40, 60, 100, 120 μg/mL[(d),(f)]	Positive	Lloyd (2012)	The MNBN cell frequencies increases were statistically significant at the top two concentrations, but only slightly exceeded the 95% range of historical controls at the highest dose. All other treated cultures fell within the normal range. The study complies with OECD TG 487.
			100, 130, 140 μg/mL[(e),(f)] 20, 23, 26 μg/mL[(d),(g)]	Negative Negative	Lloyd (2012)	The MNBN cell frequencies increases were statistically significant at the top two concentrations, but all treated cultures fell within the normal range. The study complies with OECD TG 487.
			20, 60, 70 and 80 μg/mL[(d),(f)]	Positive	Lloyd (2012)	The MNBN cell frequencies in both cultures at 20 and 70 μg/mL and in one culture at 80 μg/mL exceeded the 95th percentile of the historical control range. The study complies with OECD TG 487.

(a): With and without S9 metabolic activation.
(b): Plate incorporation method.
(c): Pre-incubation method.
(d): Without S9 metabolic activation.
(e): With S9 metabolic activation.
(f): 3-h incubation with 21-h recovery period.
(g): 24-h incubation with no recovery period.

Table D.2: Summary of *in vivo* genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] of subgroup 3.3

Chemical Name [FL-no]	Test system *in vivo*	Test object Route	Doses	Result	Reference	Comments
2-Phenylcrotonaldehyde [FL-no: 05.062]	Micronucleus assay in bone marrow	Rat Gavage	70, 350 and 700 mg/kg bw per day	Negative	Henderson (2013)	The study complies with OECD TG 474. No clear evidence of bone marrow exposure, therefore the test is inconclusive.

Appendix E – Genotoxicity Data on 2-phenylcrotonaldehyde [FL-no: 05.062] evaluated in FGE.216Rev2

Table E.1: Summary of additional *in vitro* genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] of subgroup 3.3

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
2-Phenylcrotonaldehyde [FL-no: 05.062]	Reverse Mutation test	*S. typhimurium* TA98, TA100, TA1535, TA1537 *E. coli* WP2 uvrA	1.5, 5.0, 15, 50, 150, 500, 1,500 and 5,000 μg/plate[(a),(b),(c)] 15, 50, 150, 500, 1,000, 1,500 and 5,000 μg/plate[(a),(b)]	Positive	BioReliance (2016)	Reliable without restrictions. Study performed according to OECD TG 471 and in compliance with GLP.
	Micronucleus assay with CREST staining	Human TK6 cells	20, 40, 45 μg/mL[(d),(f)] 40, 60, 80 μg/mL[(e),(f)] 5, 15, 20 μg/mL (assay 1) 16, 18, 20 μg/mL (assay 2)[(d),(g)]	Negative Negative Positive	BioReliance (2018a)	Reliable without restrictions. Study performed according to OECD TG 487 and in compliance with GLP. The given concentrations are those for the cultures that were scored for micronuclei. CREST analysis indicates that 2-phenylcrotonaldehyde induced MN by an anuegenic mechanism.

(a): With and without S9 metabolic activation.
(b): Plate incorporation method.
(c): Preliminary toxicity and mutagenicity test.
(d): Without S9 metabolic activation.
(e): With S9 metabolic activation.
(f): 4-h incubation with 23-h recovery period.
(g): 27-h incubation with no recovery period.

Table E.2: Summary of additional *in vivo* genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] of subgroup 3.3

Chemical name [FL-no]	Test system *in vivo*	Test Object Route	Doses	Result	Reference	Comments
2-Phenylcrotonaldehyde [FL-no: 05.062]	Micronucleus assay in bone marrow	Rat gavage	70, 350 and 700 mg/kg bw per day	Inconclusive	Covance (2013)	Reliable with restrictions (no clear evidence of bone marrow exposure). Study performed in compliance with GLP and according to OECD TG 474. The highest dose tested (700 mg/kg bw per day) was an estimate of the MTD according to the micronucleus study by Henderson (2013).
	Plasma bioanalysis (Micronucleus assay)[(a)]	Rat gavage	700 mg/kg bw per day	Inconclusive	Covance (2014)	CG-MSD method validated (recovery, accuracy and precision). Linearity and working range were assessed. The concentration of 2-phenylcrotonaldehyde detected was below the linearity range. Not a GLP study.
	Comet assay in duodenum	Rat gavage	175, 350, 700 mg/kg bw per day	Negative	Covance (2016)	Reliable without restrictions. Study performed in compliance with GLP and according to OECD TG 489. The dose of 700 mg/kg bw per day was an estimate of the MTD according to the micronucleus study by Henderson (2013).
	Gene mutation assay in duodenum, liver and bone marrow	Big Blue® C57BL/6 transgenic mice gavage	75, 250 and 500 mg/kg bw per day	Negative	BioReliance (2018b)	Reliable without restrictions. Study performed in compliance with GLP and according to OECD TG 488.

(a): Plasma obtained from satellite group of animals in the study by Henderson (2013) and from satellite group of animals in the study by Covance (2013).

Appendix F – Exposure

F.1. Presence in food

According to the Volatile Compounds in Food (VCF) database, 2-phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] are reported to be present in natural food sources and processed food as listed in Tables F.1, F.2, F.3 and F.4. These tables report a non-exhaustive list of foodstuff containing [FL-no: 05.062, 05.099, 05.100, 05.175]. 2-Phenyl-4-methyl-2-hexenal [FL-no: 05.222] is not reported to be present in food (VCF online database, 2022).

Table F.1: Examples of 2-phenylcrotonaldehyde [FL-no: 05.062] occurrence in foodstuff

Food item	Process	Qualitative	Quantitative (ppm)	Reference
Asparagus (Asparagus officinalis L.)	Cooked		0.01	Tressl et al. (1977)
	Raw	Yes		
Black tea	Raw	Yes		Sato et al. (1970)
	Information not available	Yes		Bricout et al. (1967)
	Microbial fermentation	Yes		Kawakami et al. (1987)
	Fermentation/Microbial fermentation	Yes		Kobayashi and Kawakami (1991)
	Brewing		0.1–0.15	Schreier and Mick (1984)
Buckwheat flour	Boiled	Yes		Yajima et al. (1983)
Cocoa butter	From roasted and unroasted beans	Yes		Carlin et al. (1982)
Cocoa liquor	Beans are roasted, shelled and finely ground into liquor	Yes		Counet et al. (2004)
				Baigrie and Rumbelow (1987)
				Muggler-Chavan and Reymond (1967)
Coffee	Roasting		0.1–0.2	Silwar et al. (1987)
	Information not available		0.6	Silwar (1982)
Dark chocolate	Produced *in house*	Yes		Afoakwa et al. (2009)
	Produced by roasted beans and conching			Owusu et al. (2012)
Milk chocolate	Storage	Yes		Ziegleder and Stojacic (1988)
Cocoa beans	Roasting		2.5–3	Silwar (1988)
	Roasting	Yes		Van Praag et al. (1968)
	Raw/Roasting	Yes		Ziegleder (1991)
Filbert hazelnut (Corylus avellana)	Roasting	Yes		Kinlin et al. (1972)
Katsuobushi (Dried bonito – variety of fish)	Drying – soup stock	Yes		Washino et al. (1989)
Macadama nut (Macadamia integrifolia)	Roasting and grinding	Yes		Crain and Tang (1975)
Barley	Malting		0.01–0.045	Kossa et al. (1979)
	Raw	Yes		Farley and Nursten (1980)
Fish meat paste, soy and rice miso	Fermentation	Yes		Giri et al. (2010)

Food item	Process	Qualitative	Quantitative (ppm)	Reference
Mushrooms	Raw		0.1	Pyysalo (1975)
	Raw and pressed into juices	Yes		Pyysalo (1976)
	Raw	Yes		Pyysalo and Honkanen (1976)
Okra (Hibiscus esculentus L.)	Raw	Yes		Ames and MacLeod (1990)
Peanut oil	Cold-pressed from raw and roasted peanuts		0.1	Brown et al. (1973)
	Roasted peanuts	Yes		Johnson et al. (1971)
Pork (liver)	Pressure Cooked	Yes		Mussinan and Walradt (1974)
Potato chips (American)	Frying	Yes		Buttery (1973)
Rice bran	Raw	Yes		Tsugita et al. (1978)
Sesame seed	Roasting	Yes		Manley et al. (1974)
Soybean	Extrusion from defatted flour	Yes		Ames and Macleod (1984)
Yeast extract (Saccharomyces cerevisiae)	Processed in different ways and supplied as powder	Yes		Ames and Elmore (1992)
	Processed in different ways and supplied as paste	Yes		Mahadevan and Farmer (2006)

Table F.2: Examples of 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] occurrence in foodstuff

Food item	Process	Qualitative	Quantitative (ppm)	Reference
Black tea leaves	Brewing	Yes		Renold et al. (1974)
Cocoa butter	Expeller pressing of heated cocoa beans	Yes		Carlin et al. (1982)
Cocoa liquor	Beans are roasted, shelled and finely ground into liquor	Yes		Magi et al. (2012)
		Yes		Baigrie and Rumbelow (1987)
Cocoa powder	Roasting		0.52–1.94	Bonvehi (2005)
Dark chocolate	Produced in house	Yes		Afoawka et al. (2009)
	Produced by roasted beans and conching	Yes		Owusu et al. (2012)
	Before and after Conching		0.496–0.546	Counet et al. (2002)
Milk chocolate	Storage	Yes		Ziegleder and Stojacic (1988)
Cocoa beans	Roasting		0.3–0.5	Silwar (1988)
	Raw/Fermentation/Roasting		1	Gill et al. (1984)
	Roasting	Yes		Van Praag et al. (1968)
	Roasting	Yes		Stofberg and Stoffelsma (1981)
	Raw/Roasting	Yes		Ziegleder (1991)
Filbert hazelnut (Corylus avellana)	Roasting	Yes		Kinlin et al. (1972)
Barley	Malting		< 0.01–0.065	Kossa et al. (1979)
		Yes		Farley and Nursten (1980)

Food item	Process	Qualitative	Quantitative (ppm)	Reference
Peppermint oil (Mentha piperita L.)	Raw	Yes		Takahashi et al. (1980)
Peanut Oil	Roasted peanuts	Yes		Johnson et al. (1971)
Pork (liver)	Pressure cooked	Yes		Mussinan and Walradt (1974)
Potato (Solanum tuberosum L.)	Frying	Yes		Carlin (1983)
Sesame seed	Roasting	Yes		Manley et al. (1974)
Wort	Boiling	Yes		Buckee et al. (1982)

Table F.3: Examples of 4-methyl-2-phenylpent-2-enal [FL-no: 05.100] occurrence in foodstuff

Food item	Process	Qualitative	Quantitative (ppm)	Reference
Black tea leaves	Brewing	Yes		Renold et al. (1974)
Cocoa butter	From roasted and unroasted beans	Yes		Carlin et al. (1982)
Cocoa powder	Roasting		0.025–0.31	Bonvehi (2005)
Milk chocolate	Storage	Yes		Ziegleder and Stojacic (1988)
Cocoa beans	Roasting		0.05–0.1	Silwar (1988)
	Roasting	Yes		Van Praag et al. (1968)
	Raw/Roasting	Yes		Ziegleder (1991)
Filbert hazelnut (Corylus avellana)	Roasting	Yes		Kinlin et al. (1972)
Barley	Malting		< 0.01–0.02	Kossa et al. (1979)
Peppermint oil (Mentha piperita L.)	Raw		6	Takahashi et al. (1980)
Potato (*Solanum tuberosum* L.)	Baking with skin	Yes		Coleman et al. (1981)
	Frying	Yes		Carlin (1983)
Potato chips (American)	Frying	Yes		Buttery (1973)
Sesame seed	Roasting	Yes		Manley et al. (1974)
Yeast extract (Saccharomyces cerevisiae)	Processed in different ways and supplied as powder	Yes		Ames and Elmore, (1992)

Table F.4: Examples of 2-phenylpent-2-enal [FL-no: 05.175] occurrence in foodstuff

Food item	Process	Qualitative	Quantitative (ppm)	Reference
Peanut	Roasting	Yes		Walradt et al. (1971)
Peanut Oil	Cold pressed	Yes		Brown et al. (1973)
Sesame seed	Roasting	Yes		Manley et al. (1974)

F.2. Uses and use levels as provided by the Flavour Industry

For each substance, normal and maximum use levels have been provided by industry (documentation provided to EFSA No. 10) according to the different food categories reported in Annex I of Regulation (EC) 1565/2000[3] or according to the EFSA Guidance on the data required for the risk assessment of flavourings to be used in or on foods (EFSA CEF Panel, 2010). For substances 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222] use levels are reported only for the food categories according to of Regulation (EC) 1565/2000. Use levels data (Table F.5) have been used to calculate the mTAMDI. Since the calculation of mTAMDI is based on food categories as reported in Annex I of Regulation (EC) 1565/2000 (Table F.6), for the substances [FL-no: 05.062, 05.099, 05.100] the highest values of the normal use levels among the subcategories have been selected.

Table F.5: Use levels reported by industry for the flavouring substances in FGE.19 subgroup 3.3

CODEX code	Food categories[a],[d]	Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)									
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			05.062		05.099		05.100		05.175[c]		05.222[c]	
01.0	Dairy products and analogues, excluding products of category 02.0		0.41	1.4	2.12	8.65	0.41	22.4	3	15	0.0002	0.0075
01.1	Milk and dairy-based drinks	200	0.25	0.56	1.56	5.43	0.19	0.53				
01.6	Cheese and analogues	40	0.37	1.4	1.64	3.21	0.41	22.4				
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	125	0.41	1.3	2.12	8.65	0.0022	0.5				
02.0	Fats and oils and fat and oil emulsions (type water-in-oil)		0.48						2	10	0.0002	0.0075
02.1	Fats and oils essentially free from water	15	0.16	1.56	5.68	17.38	0.11	5.61				
02.2	Fat emulsions mainly of type water-in-oil	15	0.16	1.56	5.68	17.38	0.11	5.61				
02.3	Fat emulsions mainly of type water-in-oil, including mixed and/or flavoured products based on fat emulsions	15	0.16	1.56	5.68	17.38	0.11	5.61				
02.4	Fat-based desserts excluding dairy-based dessert products of category 1.7	50	0.48	1.23	0.85	9	0.03	0.79				
03.0	Edible ices, including sherbet and sorbet	50	0.44	1.15	3.89	13.15	0.03	0.55	3	15	0.002	0.005
04.1.2	Processed fruit	125	0.03	0.05	0.5	1.01	0.0021	0.09	2	10	0.0002	0.0075
04.1.2.5	Jams, jellies, marmalades	30	0.26	0.26	0.78	2.69	0.0032	0.16				

CODEX code	Food categories[a],[d]	Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)									
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			05.062		05.099		05.100		05.175[c]		05.222[c]	
04.2.2	Processed vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweed and nut and seed purees and spreads (e.g. peanut butter) and nuts and seeds	200	0.03	0.13	0.05	0.46	0.12	1.5				
05.0	Confectionery		2.52						4	20	0.02	0.75
05.1	Cocoa products and chocolate products, including imitations and chocolate substitutes	40	0.97	2.64	4.18	21.24	0.23	11.25				
05.2	Confectionery, including hard and soft candy, nougats, etc., other than 05.1, 05.3 and 05.4	30	0.54	3.5	3.82	19.84	0.34	6.2				
05.3	Chewing gum	3	2.52	5.13	4.66	11.52	2.03	112				
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	35	0.11	1.05	1.65	10.41	0.2	11.2				
06.0	Cereals and cereal products, incl. Flours & starches from roots & tubers, pulses & legumes, excluding bakery		0.45						2	10		
06.3	Breakfast cereals, including rolled oats	30	0.45	2	0.81	2.1	0.61	33.6				
06.4	Pastas and noodles and like products (e.g. rice paper, rice vermicelli, soya bean pastas and noodles)	200	0.01	0.04	0.03	0.26	0.12	1.5				
06.5	Cereal and starch based desserts (e.g. rice pudding, tapioca pudding)	200	0.19	1.33	1.04	1.58	0.41	22.4				
06.6	Batters (e.g. for breading or batters for fish or poultry)	30	0.04	0.3	1.08	3.08	0.23	3				
06.7	Pre-cooked or processed rice products, including rice cakes (Oriental type only)	200	0.3	0.6	0.02	0.06	0.1	0.2				
06.8	Soya bean products (excluding soya bean products of food category 12.9 and fermented soya bean products of food category 12.10)	100	0.05	0.32	0.44	1.62	0.15	3.76				

CODEX code	Food categories[a],[d]	Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)									
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			05.062		05.099		05.100		05.175[c]		05.222[c]	
07.0	Bakery wares		1.21						5	25	0.002	0.0075
07.1	Bread and ordinary bakery wares	50	0.51	2.14	1.7	6.71	1.42	9.51				
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	80	1.21	3.3	6.81	22.32	1.84	11.14				
08.0	Meat and meat products, including poultry and game		0.02						1	5	0.0002	0.0075
08.2	Processed meat, poultry and game products in whole pieces or cuts	100	0.7	0.83	0.08	0.11	0.12	1.5				
08.3	Processed comminute meat, poultry and game products	100	0.7	0.83	0.08	0.11	0.12	1.5				
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms		0.42						1	5	0.0002	0.0075
09.2	Processed fish and fish products, including molluscs, crustaceans and echinoderms	100	0.42	0.47	4.13	12.88	0.12	1.5				
09.3	Semi-preserved fish and fish products, including molluscs, crustaceans and echinoderms	100	0.42	0.47	4.13	12.88	0.12	1.5				
09.4	Fully preserved, including canned or fermented, fish and fish products, including molluscs, crustaceans and echinoderms	100	0.42	0.47	4.13	12.88	0.12	1.5				
10.0	Eggs and egg products		0.8									
10.2	Egg products	100	0.02	0.15	0.05	0.51	0.12	1.5				
10.3	Preserved eggs, including alkaline, salted and canned eggs	100	0.02	0.15	0.05	0.51	0.12	1.5				
10.4	Egg-based desserts (e.g. custard)	125	0.8	4	6.34	21.69	0.41	22.4				
11.0	Sweeteners, including honey		0.45									
11.1	Refined and raw sugar	10			0.96	2.38						
11.2	Brown sugar excluding products of food category 11.1	10			0.69	3.39						

CODEX code	Food categories[a],[d]	Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)									
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			05.062		05.099		05.100		05.175[c]		05.222[c]	
11.3	Sugar solutions and syrups, and (partially) inverted sugars, including molasses and treacle, excluding products of food category 11.1	30	0.01	0.04	0.26	0.85	0.0016	0.08				
11.4	Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings)	30	0.45	1.45	1.29	5.5	0.0016	0.08				
11.5	Honey	15			0.5	3.01						
11.6	Table-top sweeteners, including those containing high-intensity sweeteners	1			0.004	0.02						
12.0	Salts, spices, soups, sauces, salads, protein products, etc.		2.0						2	10	0.0002	0.0075
12.2	Herbs, spices, seasonings and condiments (e.g. seasoning for instant noodles)	1	0.41	0.56	0.22	9.18	0.09	0.81				
12.3	Vinegars	15	0.38	1.75	1	3.01	0.12	1.5				
12.4	Mustards	15	0.15	0.15	2.09	5.84	0.12	1.5				
12.5	Soups and broths	200	0.27	0.31	0.07	0.11	0.06	0.76				
12.6	Sauces and like products	30	0.38	1.88	0.42	1.63	0.12	1.5				
12.7.1	Salads 120 g (e.g. macaroni salad, potato salad) excluding cocoa- and nut-based spreads of food categories	120	0.02	0.15	0.05	0.51	0.12	1.5				
12.7.2	Sandwich spreads (20 g), excluding cocoa- and nut-based spreads of food categories	20	0.02	0.15	2.32	7.58	0.12	1.5				
12.9	Protein products	15	2	4	6.09	75.17	0.58	7.5				
12.10	Fermented soya bean products	40	1	1	7.25	25.01	0.12	1.5				
13.0	Foodstuffs intended for particular nutritional uses		0.3						3	15		
13.3	Dietetic foods intended for special medical purposes (excluding food products of category 13.1)	200	0.3	2.05	0.58	1.09	0.16	11.21				

CODEX code	Food categories[a],[d]	Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)									
			Normal	Max	Normal	Max	Normal	Max	Normal	Max	Normal	Max
Flavouring substances FL-no:			05.062		05.099		05.100		05.175[c]		05.222[c]	
13.4	Dietetic formulae for slimming purposes and weight reduction	200	0.3	2.05	0.58	1.09	0.16	11.21				
13.5	Dietetic foods (e.g. supplementary foods for dietary use), excluding products of food categories 13.1–13.4 and 13.6	200	0.3	2.05	0.58	1.09	0.16	11.21				
13.6	Food supplements	5			1.14	6.6	0.07	0.57				
14.1	Non-alcoholic ('soft') beverages	300	0.55	2.29	1.54	9.87			2	10	0.02	0.75
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts		0.5		0.11	0.11			4	20	0.02	0.75
14.2.1	Beer and malt beverages	300	0.13	0.13	0.47	1.29	0.0016	0.08				
14.2.2	Grape wines	150	0.23	0.23	2.47	8.39	0.0016	0.08				
14.2.3	Mead	150	0.5	0.5	4.84	16.71	0.0016	0.08				
14.2.4	Spirituous beverages	30	0.4	1.44	0.52	9.45	0	0.08				
15.0	Ready-to-eat savouries	30	2.53[e]	2.29[e]	3.64	10.04	0.78	1.79	5	25	0.0002	0.0075
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01.0–15.0	300	0.08	0.34	0.55	2.25			2	10	0.0002	0.0075

(a): Most of the categories reported are the subcategories of Codex GSFA (General Standard for Food Additives, available at http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) used by the JECFA in the SPET technique (FAO/WHO, 2008).

(b): For Adults. In case of foods marketed as powder or as concentrates, occurrence levels must be reported for the reconstituted product, considering the instructions reported on the product label or one of the standard dilution factors established by the JECFA (FAO/WHO, 2008):

- 1/25 for powder used to prepare water-based drinks such as coffee, containing no additional ingredients,
- 1/10 for powder used to prepare water-based drinks containing additional ingredients such as sugars (ice tea, squashes, etc.),
- 1/7 for powder used to prepare milk, soups and puddings,
- 1/3 for condensed milk.

(c): Only use levels for 'main' food categories have been provided (see Table F.6).

(d): Only food categories for which use has been reported are included in the table.

(e): The Panel noted that the normal and maximum use levels for this food category for [FL-no: 05.062] are not plausible. However, use level of 2.29 mg/kg was provisionally used for the calculation of mTAMDI.

Table F.6: Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000[3] into the seven SCF food categories used for TAMDI calculation (SCF, 1995)

Key	Food categories according to Commission Regulation 1565/2000	Distribution of the seven SCF food categories		
	Food category	**Foods**	**Beverages**	**Exceptions**
01.0	Dairy products, excluding products of category 02.0	Foods		
02.0	Fats and oils, and fat emulsions (type water-in-oil)	Foods		
03.0	Edible ices, including sherbet and sorbet	Foods		
04.1	Processed fruit	Foods		
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	Foods		
05.0	Confectionery			Exception a
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	Foods		
07.0	Bakery wares	Foods		
08.0	Meat and meat products, including poultry and game	Foods		
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	Foods		
10.0	Eggs and egg products	Foods		
11.0	Sweeteners, including honey			Exception a
12.0	Salts, spices, soups, sauces, salads, protein products, etc.			Exception d
13.0	Foodstuffs intended for particular nutritional uses	Foods		
14.1	Non-alcoholic ('soft') beverages, excl. dairy products		Beverages	
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts			Exception c
15.0	Ready-to-eat savouries			Exception b
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 01.0–15.0	Foods		

F.3. Intake data from intended use

Annual production volumes of the flavouring substances as surveyed by industry are used to calculate the 'Maximised Survey-derived Daily Intake' (MSDI) assuming that the production figure only represents 60% of the use in food, due to underreporting and that 10% of the total EU population are consumers (SCF, 1999).

Use levels for 2-phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222] provided by industry (documentation provided to EFSA No. 10) and listed in Table F.5 have been used to calculate the 'modified Theoretical Added Maximum Daily Intake' (mTAMDI).[6] When use levels for subcategories are reported according to the EFSA Guidance on the data required for the risk assessment of flavourings (EFSA CEF Panel, 2010), the highest value of the normal use levels among the subcategories has been selected as the value for each main category that is used for the calculation of mTAMDI.

The MSDI and mTAMDI exposure estimates are given in Table F.7.

Table F.7: Estimated exposure to 2-phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222]

FL-no	Chemical name	EU MSDI µg/capita per day	mTAMDI µg/person per day
05.062	2-phenylcrotonaldehyde	1.7	466.6
05.099	5-methyl-2-phenylhex-2-enal	15	1749.6
05.100	4-methyl-2-phenylpent-2-enal	0.34	285.9
05.175	2-phenylpent-2-enal	0.011	1,643
05.222	2-phenyl-4-methyl-2-hexenal	3	7.5

[6] mTAMDI estimation is based on an approach used by the SCF up to 1995 (SCF, 1995) and is calculated on the basis of standard portions and normal use levels for flavoured beverages and foods in general, with exceptional levels for particular foods.